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Calcium Waves Propagate through Radial Glial Cells and Modulate Proliferation in the Developing Neocortex

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Summary

The majority of neurons in the adult neocortex are produced embryonically during a brief but intense period of neuronal proliferation. The radial glial cell, a transient embryonic cell type known for its crucial role in neuronal migration, has recently been shown to function as a neuronal progenitor cell and appears to produce most cortical pyramidal neurons. Radial glial cell modulation could thus affect neuron production, neuronal migration, and overall cortical architecture; however, signaling mechanisms among radial glia have not been studied directly. We demonstrate here that calcium waves propagate through radial glial cells in the proliferative cortical ventricular zone (VZ). Radial glial calcium waves occur spontaneously and require connexin hemichannels, $P2Y_1$ ATP receptors, and intracellular IP_3-mediated calcium release. Furthermore, we show that wave disruption decreases VZ proliferation during the peak of embryonic neurogenesis. Taken together, these results demonstrate a radial glial signaling mechanism that may regulate cortical neuronal production.

Introduction

Although neuronal production does not readily occur in the adult mammalian neocortex, the embryonic cortical environment is conducive to growth and reorganization and is characterized by high rates of new neuron production. The specific mechanisms underlying this unique proliferative period are not fully understood. Interestingly, however, there is a transient population of dividing radial glial cells present during the embryonic period of neurogenesis (Rakic, 2003). Although previously believed to play a structural role in guiding neuronal migration, radial glial cells have recently been shown to also function as neuronal progenitor cells (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001). In fact, radial glia appear to be responsible for the production of most excitatory cortical neurons during development (Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004) and are also present in regions of the adult nonmammalian brain that are known to support neurogenesis throughout life (Alvarez-Buylla et al., 2001; Zupanc and Clint, 2003; Garcia-Verdugo et al., 2002; Weissman et al., 2003). Radial glial cells are thus important neuronal progenitor cells and may in part underlie the unique proliferative capacity of the embryonic cortex.

The centralization of neurogenesis and migrational guidance within the radial glial cell raises the intriguing possibility of efficient regulatory control, with the activity of a single cell type potentially affecting both neuronal production and overall cortical architecture. Since some human developmental disorders associated with cortical malformations involve defects in neuronal migration as well as neuronal production, radial glial cell modulation could have important clinical implications. Moreover, the unique morphology of the radial glial cell (Bentivoglio and Mazzarello, 1999), with a cell body in the proliferative ventricular zone (VZ) and a radial fiber that contacts distant neurons in the developing cortical plate (CP), may allow for direct interaction between progenitor cells and neuronal progeny throughout development.

While recent findings have expanded our understanding of radial glial cell function, little is known about possible radial glial cell regulation. One potential mechanism for modulating the radial glial cell cycle could involve calcium increases (Owens and Kriegstein, 1998), which are known to tightly regulate proliferation in many cell types (Berridge, 1995). Calcium transients in VZ cells have been proposed to play a role in cell division (Owens and Kriegstein, 1998), neuronal differentiation (Gu and Spitzer, 1997), and neuronal migration (Komuro and Rakic, 1996) and could be propagated easily throughout the highly gap junction-coupled VZ (Lo Turco and Kriegstein, 1991; Bittman et al., 1997). Importantly, astrocytes, into which many radial glial cells transform postnatally (Rakic, 2003), are known to sustain robust calcium waves in culture (reviewed in Haydon, 2001). Since radial glia and astrocytes are developmentally related (Alvarez-Buylla et al., 2001; Gotz et al., 2002), we examined whether calcium dynamics might contribute to functional signaling mechanisms in radial glial cells.

We demonstrate here that spontaneous intercellular calcium waves propagate through radial glial cells in the embryonic VZ. These waves, which can also occur in response to electrical or mechanical stimulation, are mediated by extracellular ATP and involve the release of calcium from IP_3-sensitive intracellular stores. Spontaneous calcium waves appear to be initiated by the opening of radial glial connexin hemichannels in a cell cycle-specific manner. Furthermore, the waves become most robust during the peak of neuronal production in the VZ, and we show that disrupting the calcium wave signaling pathway at this stage of development reduces VZ proliferation. Considering recent evidence that radial glial cells produce neurons (Malatesta et al., 2000; Noctor et al., 2001), this dynamic radial glial signaling mechanism may be involved in regulating rates of cortical neuron production.

Results

Spontaneous Calcium Waves Propagate through the Embryonic Ventricular Zone

To begin to investigate calcium signaling in radial glial cells, we loaded acute coronal brain slices and cortical

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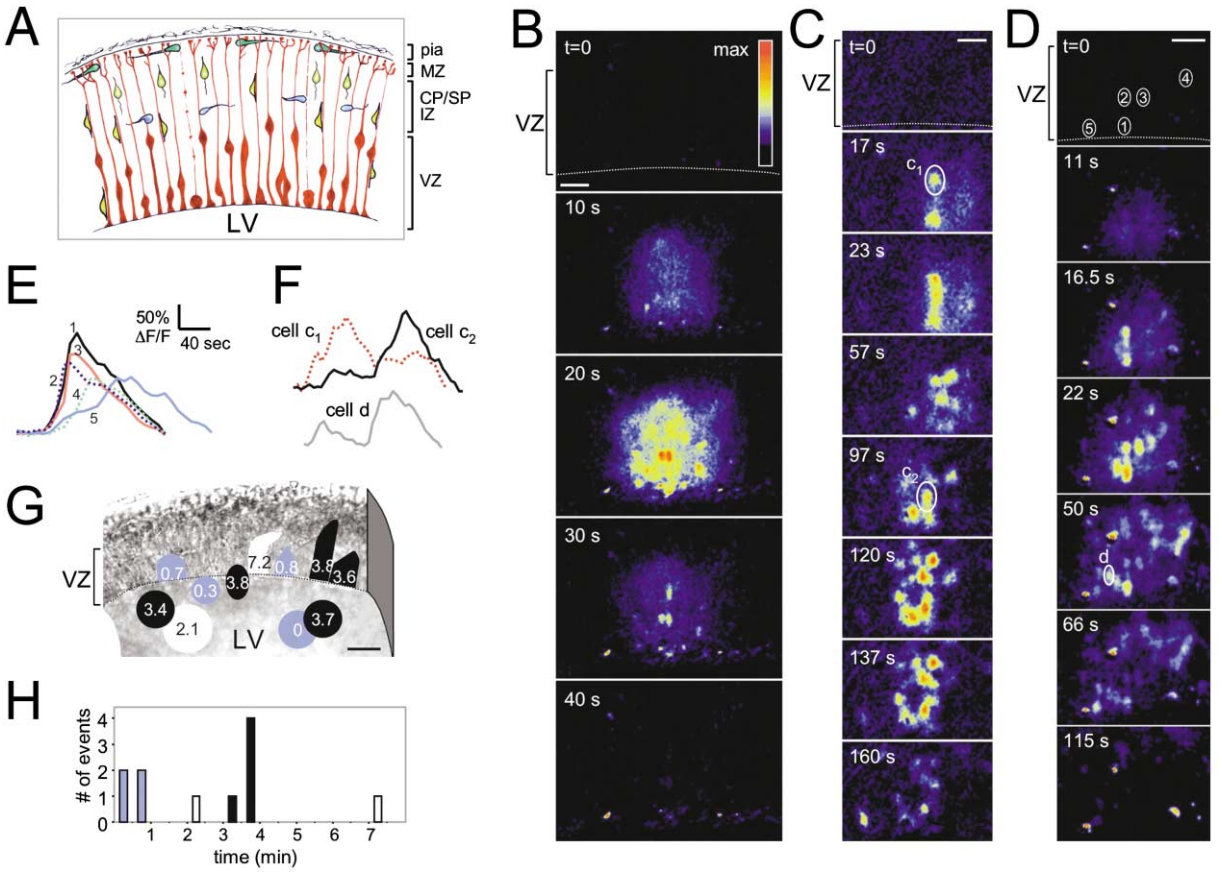

Figure 1. Spontaneous Calcium Waves Propagate through the Embryonic Ventricular Zone

(A) Schematic drawing illustrates cortical anlage at embryonic day 16 (E16). LV, lateral ventricle; VZ, ventricular zone; CP, cortical plate; IZ, intermediate zone; SP, subplate; MZ, marginal zone.

(B–D) Individual spontaneous waves occurring within the VZ. (VZ is delineated at the left of each initial frame.) Waves initiate in VZ cells and propagate dorsally and medial/laterally. Scale bar, 25 μm.

(E) Selected individual cell transients (1–5) from (D) are represented as ΔF/F traces. Calcium levels in cells 4 and 5 (furthest from the initiation point) increase as levels in cells 1–3 decay.

(F) Some cells show spontaneously oscillating calcium levels, for example, cells c_1 and c_2 from (C) and cell d from (D).

(G) Spontaneous waves occur in a pattern of temporal clusters. This partially schematized image represents a three-dimensional field from an E16 coronal slice, with the ventricular surface sloping away in the lower part of the image. In this field, 11 waves were imaged in 8 min of observation. Shapes are drawn to approximate the spatial extent of each wave. The first four events (blue) occurred within the first minute, while five later events (black) were clustered within the fourth minute of observation. White events did not appear to occur within temporal clusters. Several events occurred in endfeet contacting the ventricular surface. Scale bar, 50 μm.

(H) Temporal clustering is shown in graphical form with the number of events plotted as a function of time (0.5 min bins).

explants from embryonic day (E) 16–17 rat with the calcium indicator Fluo-3. At this stage of development, the cortical anlage consists of a proliferative VZ bordering the lateral ventricle, a thin marginal zone (MZ) at the dorsal margin just below the pia, and a developing CP and intermediate zone in-between (Figure 1A; Bayer and Altman, 1991).

Using time-lapse calcium imaging, we observed spontaneous calcium waves occurring in clusters of VZ cells (Figures 1B–1D). Spontaneous waves were largely restricted to cells within the VZ; only 11 out of 213 events spread to cells outside of the VZ. Most waves included at least one cell at the top of the VZ, in the S phase zone (92.3% of waves imaged in coronal sections), and at least one cell at the ventricular surface, in the M phase zone (95.3%). Spontaneous calcium waves propagated at an average velocity of 6.0 ± 0.07 μm/s (four slices) and occurred with a frequency of 1.1 events/min/mm (263.8 min total, 18 hemispheres aged E16–E17). (In our previous studies, wave-like events in the VZ occurred too infrequently for detailed analysis [Owens and Kriegstein, 1998]. To maximize our detection of spontaneous VZ events here, we borrowed techniques established from work on astrocyte calcium waves [see Experimental Procedures; Zanotti and Charles, 1997; Cotrina et al., 1998; Stout et al., 2002].) Individual cell calcium transients lasted an average of 48.4 ± 8.7 s (n = 31 cells selected from seven waves, six hemispheres), with some cells displaying oscillating calcium transients (Figures 1E and 1F). Recurrent waves were not observed at the same site, although nearby waves were often generated sequentially, in clusters radiating both spatially and temporally from one origin (Figures 1G and 1H). Spontaneous calcium waves could travel

over 75 μm and include up to at least 35 individual cells. Calcium waves occurred in all regions of the cortical VZ, from medial to lateral and rostral to caudal.

We next tested whether the calcium waves could be reproduced consistently using electrical or mechanical stimulation and found that, in regular ACSF, brief stimulation applied focally in the MZ led to a robust intercellular calcium wave in the underlying VZ (Supplemental Figures S1A and S1B [http://www.neuron.org/cgi/content/full/43/5/647/DC1]). These waves involved many more cells than the spontaneous events. Several seconds following a single electrical or mechanical MZ stimulation, a slow wave began in the VZ directly below the electrode and traveled tangentially within the VZ up to a distance of 350 μm (Supplemental Figure S1C; average distance = 158.9 ± 10.5 μm, n = 56 slices). The delay between stimulation and wave initiation in the VZ indicated that the initiating signal traveled radially from the stimulation site at an average velocity of 5.8 ± 0.4 μm/s (n = 10 slices). Waves then traveled medially/laterally within the VZ at an average velocity of 10.9 ± 0.4 μm/s (Supplemental Figure S1D; n = 36 slices). Propagation kinetics were thus similar to the spontaneous waves. Individual cell calcium transients in stimulated waves lasted on average 42.6 ± 1.8 s (n = 69 selected cells), also similar to the spontaneous events. Electrically or mechanically stimulated waves were also restricted to cells within the VZ; most cell bodies in the overlying IZ or CP did not exhibit detectable calcium increases (see, for example, Supplemental Figure S1E). However, calcium signals could sometimes be seen in thin radial fibers emanating from VZ cells and coursing through the IZ toward the pia (Supplemental Figures S1E and S1F). In summary, calcium waves propagate spontaneously among VZ cells and can be reproduced by using a brief pulse of electrical or mechanical stimulation.

To address the physiological relevance of the waves, we tested whether calcium waves could be initiated in partial whole-brain preparations (Supplemental Figure S2 and Movie 1 [http://www.neuron.org/cgi/content/full/43/5/647/DC1]). Mechanical stimulation at the surface of the brain could trigger a calcium wave that traveled over 200 μm through the cerebral hemisphere, raising the possibility that mechanical stimulation could activate calcium waves in utero.

Calcium Waves Require a Diffusible Signal and Functional Connexin Hemichannels

Since VZ cells are highly gap junction coupled to one another (Lo Turco and Kriegstein, 1991; Bittman et al., 1997; Nadarajah et al., 1997), we examined whether VZ calcium waves are mediated intercellularly through gap junctions or extracellularly by a diffusible signal. When we separated two cortical slices by up to 130 μm and stimulated one slice (slice 1, Supplemental Figure S3A [http://www.neuron.org/cgi/content/full/43/5/647/DC1]), we found that a wave could spread across the gap to the unstimulated slice (slice 2, n = 6/6, Supplemental Figures S3B and S3C), indicating that a diffusible signal likely mediates wave propagation. To rule out the unlikely possibility that the second slice was stimulated directly by current spread from the electrode, we repeated the experiment in the absence of the stimulated slice, placing the electrode less than 50 μm away from slice 2, and consistently found no calcium response. These data suggest that VZ waves are mediated by a diffusible signal. We next tested whether the propagation of stimulated VZ waves requires functional gap junctions and found that standard techniques used to close gap junctions—acidification (pH 6.5), carbenoxolone (100 μM), or octanol (1 mM)—did not abolish stimulated VZ waves (Supplemental Figures S3D and S3E). However, under these conditions, waves were slightly smaller than paired control waves (distance traveled = 80.2% ± 16.2% of control, $p > 0.2$, n = 8; wave amplitude = 72.2% ± 12.1% of control, $p < 0.05$; n = 8), suggesting that gap junctions may contribute to, but are not required for, stimulated calcium wave propagation.

Conflicting results regarding the role of gap junctions in astrocyte waves have been partially explained by the identification of a role for unapposed connexin hemichannels: plasma membrane channels that are gated by mechanisms similar to functional gap junctions but which allow release into the extracellular space (Bennett et al., 2003; Goodenough and Paul, 2003). In astrocytes, spontaneous opening of a hemichannel has been suggested to cause release of factors that act upon neighboring cells, initiating a local calcium wave (Cotrina et al., 1998; Arcuino et al., 2002; Stout et al., 2002). In our system, we could address the initiation mechanism by comparing the stimulated and the spontaneous waves, since stimulated waves appear to recapitulate spontaneous waves while bypassing the spontaneous initiation event. We therefore tested whether spontaneous waves occur in the presence of connexin hemichannel inhibitors. We found that spontaneous waves were effectively abolished in the presence of carbenoxolone (100 μM; Figure 2A; two events in 63.2 min, four hemispheres; frequency = 0.046 events/min/mm) or flufenamic acid (FFA; 50 μM; Figure 2A; zero events in 83.3 min, eight hemispheres). Since connexin inhibitors largely abolish spontaneous waves while having little effect on stimulated waves, these data support the possibility that hemichannels may be specifically involved in spontaneous VZ wave initiation. However, the dramatic effects of carbenoxolone and flufenamic acid upon spontaneous waves could be due to the blockade of functional gap junctions rather than unapposed hemichannels per se. Importantly, spontaneous wave frequency could be manipulated by adjusting extracellular calcium levels (<0.03 waves/min in 2 mM calcium versus 1.1 waves/min in zero calcium), a technique used to target and control the gating of hemichannels (Zanotti and Charles, 1997; Cotrina et al., 1998; Arcuino et al., 2002; Stout et al., 2002). These results demonstrate that several manipulations known to gate connexin hemichannels directly modulate spontaneous wave frequency and suggest that hemichannels are involved in the initiation of spontaneous VZ calcium waves. To confirm the presence of hemichannels in VZ cells, we made use of the small fluorescent dyes Lucifer yellow (MW 457) and Alexa-594 hydrazide (MW 758), which can pass through open hemichannels and effectively load active cells during short incubation periods. We incubated slices in the presence of Lucifer yellow (1 mM), then imaged dye uptake following fixation. We found that multiple radial

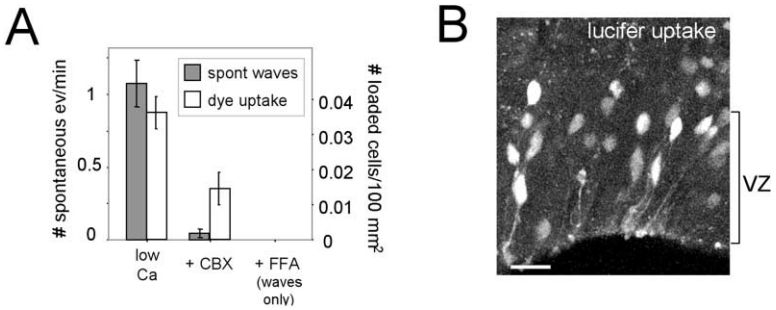

Figure 2. Connexin Hemichannels Are Involved in VZ Calcium Waves

(A) Both spontaneous calcium waves and dye uptake are modulated by changes in hemichannel gating. Spontaneous waves (dark bars) were virtually abolished when connexin channel inhibitors carbenoxolone (CBX; 100 μM) or flufenamic acid (FFA; 50 μM) were added. Dye uptake (white bars) is also decreased in the presence of carbenoxolone. (Note that dye uptake studies were not done in the presence of FFA.)

(B) During 30 min incubation periods, slices incubated in 1 mM Lucifer yellow take up dye in bipolar radial VZ cells. Scale bar, 20 μm.

cells within the VZ loaded with dye during the incubation period (Figure 2B). Dye uptake was blocked by methods known to gate connexin hemichannels, namely with connexin-specific inhibitors or increases in extracellular calcium levels, suggesting that dye uptake was mediated by hemichannels and that connexin hemichannels are present in VZ cells (Figure 2A; low calcium: $3.6 \times 10^{-2} \pm 1.3 \times 10^{-2}$ cells/100 μm^2; ACSF: $5.5 \times 10^{-3} \pm 0.7 \times 10^{-3}$ cells/100 μm^2; low calcium plus carbenoxolone: $1.4 \times 10^{-2} \pm 0.5 \times 10^{-2}$ cells/100 μm^2; $n = 5$ slices each condition). Importantly, calcium wave regulation appeared identical to the regulation of hemichannels themselves, supporting a hemichannel initiation mechanism for the spontaneous VZ waves. We repeated the dye-uptake experiments in cortical explants and in vivo by injecting Lucifer yellow intraventricularly into developing embryos and found that radial cells loaded in both preparations, suggesting that dye uptake is not restricted to slice preparations. We also included extracellular Alexa during spontaneous calcium wave imaging and found that cells within (7/12 waves) or bordering (10/12 waves) the extent of spontaneous waves loaded with dye, consistent with the possibility that waves may be initiated by hemichannel openings.

Calcium Waves Are Mediated by ATP

We next explored the mechanism for calcium wave propagation. Since our experiments above indicated a role for a diffusible factor, we tested potential extracellular signals. Several neurotransmitters are present in the developing VZ at this stage and are possible candidates for a diffusible propagating signal, including glutamate, GABA (LoTurco et al., 1995), and taurine (Flint et al., 1998). We stimulated slices in the presence of a variety of neurotransmitter receptor antagonists to identify signals involved. A combination of glutamate, GABA, and glycine receptor antagonists (APV, 100 μM; DNQX, 20 μM; bicuculline, 20 μM; strychnine, 30 μM) slightly attenuated but did not block the waves (Figure 3A, wave amplitude: 86.4% ± 4.4% of control, $p < 0.05$; distance: 68.1% ± 6.9% of control, $p < 0.05$; $n = 5$). In addition, the waves were not affected by the sodium channel blocker tetrodotoxin (TTX, 1 μM, Figure 3A; wave amplitude: 95.2% ± 12.1% of control, $p > 0.3$; distance: 101.3% ± 10.1% of control, $p > 0.6$; $n = 6$ slices) or antagonists of muscarinic acetylcholine receptors (10 μM atropine; wave amplitude: 125.9% ± 12.8% of control, $p > 0.2$; $n = 2$ slices), metabotropic glutamate receptors (100 μM MCPG; wave amplitude: 89.5% ± 10.6% of control, $p > 0.5$; $n = 2$ slices), or adenosine receptors (10 μM CPT and 2 μM DMPX).

In contrast, the presence of adenosine-5′-triphosphate (ATP) receptor antagonists affected the stimulated waves dramatically (Figures 3B–3D). The nonselective ATP receptor antagonist suramin (100 μM) and the metabotropic ATP receptor antagonist MRS2179 (10 μM, specific for the $P2Y_1$ receptor subtype) both significantly decreased the stimulated calcium waves in the VZ (Figures 3B–3D; suramin wave amplitude: 13.0% ± 1.9% of control, $p < 0.01$; suramin distance: 18.7% ± 8.2% of control, $p < 0.005$; $n = 7$ slices; MRS2179 wave amplitude: 33.1% ± 4.3% of control, $p < 0.005$; MRS2179 distance: 29.0% ± 5.5% of control, $p < 0.005$; $n = 5$ slices). In addition, the ATP receptor antagonist PPADS (45 μM), which is thought to block ionotropic and some metabotropic receptor subtypes, produced a small but significant reduction in the distance propagated (Figure 3C; PPADS distance: 75.1% ± 4.9% of control, $p < 0.05$; PPADS wave amplitude: 72.2% ± 17.0% of control, $p > 0.2$; $n = 5$ slices). These results suggest that the calcium waves may be largely mediated by extracellular ATP (or an ATP analog) acting at metabotropic ATP receptors. Although we do not rule out the possibility that ionotropic ATP receptors may be involved, the relatively slow rate of wave propagation, coupled with the block by MRS2179, a metabotropic $P2Y_1$-specific receptor antagonist, suggests that metabotropic ATP receptors play the predominant role.

We next monitored spontaneous activity in the presence of purinergic receptor antagonists in order to test whether extracellular ATP was involved in spontaneous calcium waves as well. Indeed, we found that spontaneous waves were completely abolished by either suramin (100 μM; zero events in 67.2 min, seven hemispheres) or MRS2179 (10 μM; zero events in 68.3 min, four hemispheres; Figure 3E). This indicated that, in addition to mediating electrically or mechanically stimulated calcium waves, ATP receptors were crucial for the initiation or propagation of the spontaneous calcium waves as well. Importantly, while ATP receptor antagonists abolished coordinated wave activity, random calcium transients in individual VZ cells (Owens and Kriegstein, 1998) persisted.

ATP Acts Selectively on VZ Cells

If extracellular ATP is involved in VZ wave propagation, VZ cells should express ATP receptors and respond to

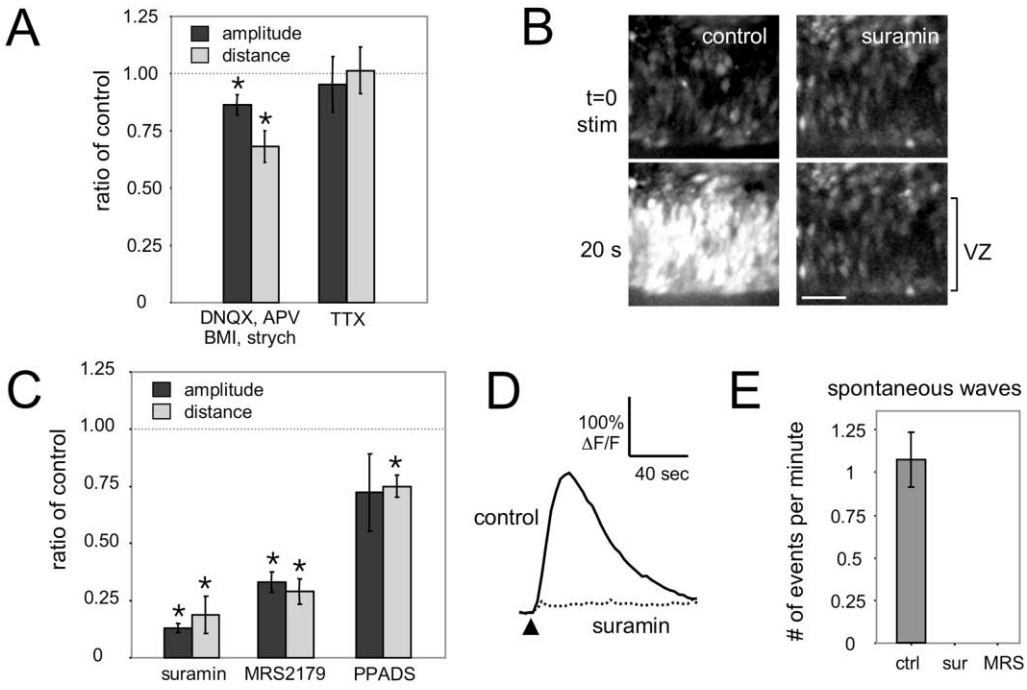

Figure 3. VZ Calcium Waves Are Mediated by Extracellular ATP

(A) Antagonists of GABA, glutamate, and glycine receptors slightly attenuated but did not block stimulated waves. Histograms are represented as average ratios of experimental waves normalized to a paired control wave within the same slice. A cocktail of DNQX (20 μM), APV (100 μM), bicuculline (20 μM), and strychnine (30 μM) slightly decreased stimulated wave amplitude (ΔF/F) and distance traveled (μm measured tangentially within the VZ). TTX (1 μM) had no effect.

(B) ATP receptor antagonists abolish stimulated VZ calcium waves. Control wave is shown in left panels at time = 0 (stim) and 20 s, the peak of the wave. Stimulated waves are abolished in the presence of the nonselective ATP receptor antagonist suramin (100 μM, right panels). Scale bar, 35 μm. Shown quantitatively in (D).

(C) ATP receptor antagonists suramin (100 μM) and MRS2179 (10 μM, $P2Y_1$-specific ATP receptor antagonist) significantly disrupted VZ calcium waves, while PPADS (45 μM, largely ionotropic ATP receptor antagonist) had a partial effect.

(D) Quantification of (B), suramin blockade of stimulated wave.

(E) Spontaneous waves are abolished in the presence of ATP receptor antagonists suramin (100 μM) or MRS2179 (10 μM).

exogenous ATP. Consistent with this prediction, direct application of ATP (1–10 μM) to coronal E16 slices resulted in robust calcium increases in VZ cells, similar to those that we observed during calcium waves (Figure 4A). As expected, the ATP receptor antagonist suramin blocked this response (Figure 4B). ATP also caused calcium increases in some subpial cells (Grafstein et al., 2000; L.I., unpublished data). No response was observed in the IZ or CP, through which newborn neurons are migrating at this stage. However, as with the stimulated waves, calcium increases could sometimes be seen in fibers emanating from VZ cells and coursing through the IZ. Importantly, both ATP and the metabotropic ATP receptor agonist 2-methylthioadenosine triphosphate (2-MeS-ATP) were desensitizing, such that subsequent waves could not be induced for many minutes following ATP exposure (Figures 4C and 4D). This is consistent with the waves being mediated by metabotropic ATP receptors, since ATP is known to desensitize P2Y receptors (Cotrina et al., 1998). We next directly applied glutamate (50–300 μM, Figure 4E), which did not affect calcium levels in the VZ but caused fast calcium increases in CP cells, most likely neurons with calcium-permeable glutamate receptors or voltage-gated calcium channels. These results indicate that VZ cells respond selectively to ATP, while neurons in the cortical plate do not.

The ATP receptor family is composed of a group of ionotropic P2X receptors and a group of metabotropic, G protein-coupled P2Y receptors (Ralevic and Burnstock, 1998). There are at least six subtypes of each group of receptor, and although specific agonists and antagonists for each subtype do not exist, one can determine which is most likely present by using an array of compounds to establish a receptor's pharmacological profile. We therefore applied various ATP analogs (all 10 μM unless specified) in order to determine which ATP receptor subtype/s are involved in VZ wave propagation (Figure 4F). We found positive responses to ATP (1–10 μM, n = 5), ADP (n = 7), 2-MeS-ATP (n = 5), and 2-chloroadenosine triphosphate (2Cl-ATP, n = 2), with a very slow response to benzoylbenzoyl-ATP (BzATP, n = 2, average response time 43.4 s as compared to 9.2 s for all others). Application of adenosine (100 μM, n = 6), uridine-5′triphosphate (UTP, n = 6), guanosine 5′-triphosphate (GTP, n = 5), or α,β-methylene-ATP (α,β-ATP, n = 3) produced little or no response. This agonist profile, coupled with the antagonist experiments from the previous section, is most compatible with the presence of the $P2Y_1$ metabotropic ATP receptor subtype ($P2Y_1R$; Ralevic and Burnstock, 1998), although the data do not rule out a small role for other ATP receptor subtypes as well. To confirm the presence of $P2Y_1R$

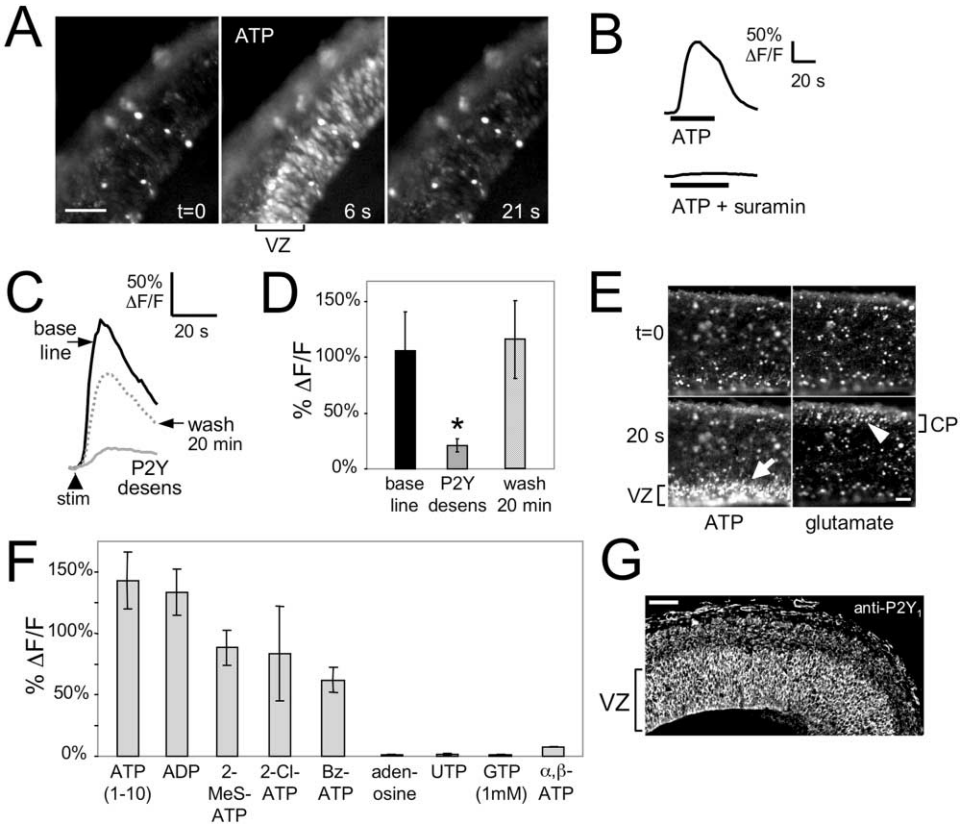

Figure 4. ATP Receptors Are Present on VZ Cells

(A) Direct application of ATP (1 μM) to coronal E16 slices causes robust calcium increases selectively in VZ cells. This effect is shown graphically ([B], upper panel) for a 200 μm wide region directly below the drug applicator and is blocked by suramin ([B], lower panel). (C and D) Application of ATP and ATP analogs desensitizes the response to wave stimulation. (C) Average ΔF/F shown in a region of the VZ following electrical stimulation (solid black trace) is greatly reduced (gray trace) following a 2–3 min exposure to 2-MeS-ATP, a metabotropic (P2Y) ATP receptor-specific ATP analog. Desensitization recovers following a 20 min wash with regular ACSF (dotted trace). (D) Five P2Y desensitization experiments were averaged for the data shown. Washout was analyzed for a subset of three experiments. (E) Application of ATP to E17 slice causes selective calcium increases in the VZ (left panels, arrow), while glutamate (100 μM) activates cells within the developing CP (right panels, arrowhead). (F) ATP analogs known to act at various ATP receptor subtypes were applied to coronal slices to determine which subtype is present in the VZ. (See Experimental Procedures and text.) The agonist profile indicates involvement of $P2Y_1R$. (G) Immunohistochemistry for the $P2Y_1$ ATP receptor subtype reveals a band of expression in the E16 VZ. Scale bar, 60 μm (A), 40 μm (E), and 50 μm (G).

in VZ cells, we used immunohistochemical staining for $P2Y_1R$ at E16 and observed a distinct band of positive staining in the VZ (Figure 4G). These results provide further evidence that extracellular ATP signaling may underlie calcium wave propagation in the VZ.

Radial Glial Cell Calcium Waves

Which cells participate in the calcium waves? Waves are restricted to the VZ—the region of the developing cortex where radial glial cell bodies are found (Rakic, 2003). In fact, recent work demonstrates that the vast majority of VZ cells during late embryonic cortical development are radial glia (Gotz et al., 2002; Noctor et al., 2002; Malatesta et al., 2003). This finding, coupled with our observation that calcium increases also occur in radial fibers, suggests that radial glia may be involved in the calcium waves. Furthermore, radial glial cells are known to express connexin channels (Bittman et al., 1997; Nadarajah et al., 1997; Bittman and LoTurco, 1999), which appear to be involved in VZ wave initiation.

To address directly the involvement of radial glia, we used a combined approach of imaging and electrophysiological recording techniques to assess the identity of cells participating in calcium waves. First, we randomly selected and recorded from VZ cells in acute E16 coronal slices using patch electrodes in the on-cell configuration (Figure 5A). This configuration maintains a gigaseal on a selected cell body but avoids dialyzation of the intracellular calcium indicator. Using this configuration, we determined whether the selected cell participated in a calcium wave by monitoring the cell's calcium levels during VZ stimulation with direct application of ATP (Figures 5B and 5D). Following stimulation, we applied suction to rupture the underlying membrane patch and examined cell morphology by filling the recorded cell with a fluorescent Alexa derivative dye (1.14 kd) that was contained in the pipette solution (Figure 5C). This dye does not readily pass through gap junctions and allowed us to selectively visualize the morphology of the stimulated cell. We found that the majority of the VZ cells displaying calcium increases in response to ATP had radial glial morphology (67%). Figure 5 illustrates

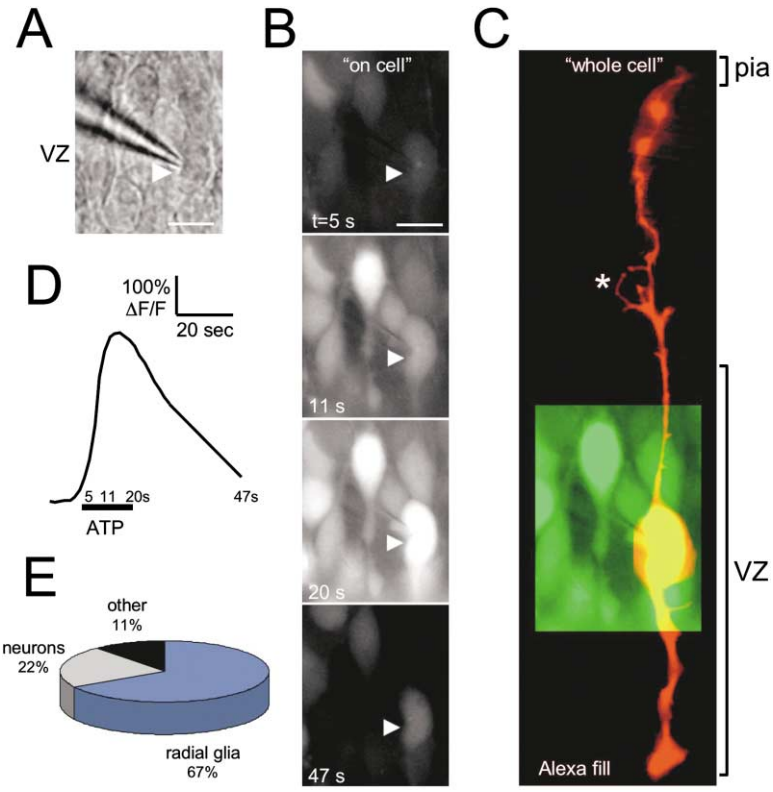

Figure 5. Radial Glial Cells Participate in VZ Calcium Waves

(A) Bright-field image shows a patch electrode on the selected cell (arrowhead) for recording. (B) Fluo-3 imaging in the "on-cell" recording configuration demonstrates that the selected cell responds during ATP application. Application began at t = 0. By 20 s, there was a robust calcium increase within the selected cell, shown quantified in (D). (C) Following stimulation, the membrane patch was ruptured to obtain the "whole-cell" recording configuration, and the cell was filled with Alexa-594-biocytin, contained within the pipette solution. Dye fill reveals the morphology of a radial glial cell, with pial fiber, enlarged ventricular foot process, and intermediate branch onto blood vessel (asterisk). (E) The majority of cells responding to ATP exhibit radial glial morphology. Of all cells responding to ATP (27/38 cells), 67% had radial glial morphology, 22% had neuronal morphology, and 11% had other morphology (see Experimental Procedures). Scale bar, 10 μm (A and B), 13 μm (C).

an example of a radial glial cell that responded to ATP. A minority of the VZ cells responding to ATP had either neuronal morphology (22%) or unknown morphology (11%) (Figure 5E; see Experimental Procedures for criteria). However, ATP does not appear to cause calcium increases in neurons migrating out of the VZ, since neither ATP application nor wave stimulation elicited a detectable calcium increase in IZ cells, largely migrating neurons at this age. We repeated these experiments using electrically or mechanically stimulated waves in place of ATP application and found that cells with radial glial morphology also participated in induced calcium waves. These results demonstrate that radial glia respond to ATP and strongly suggest that radial glial cells participate in the calcium waves that we observe in the VZ.

Waves Require IP_3-Mediated Calcium Release but Not Extracellular Calcium

Cytosolic calcium elevations can be caused either by influx from the extracellular space or by release from intracellular calcium stores located within the endoplasmic reticulum (ER) (Berridge et al., 2000). To determine the source of calcium increase in the stimulated waves demonstrated here, we first removed calcium from the extracellular solution and replaced it with the calcium chelator EGTA (2 mM). Stimulated calcium waves were not attenuated under these conditions (Figure 6A, Ca-free distance: 177.0% ± 43.4% of control, $p > 0.3$; Ca-free wave amplitude: 122.7% ± 27.1% of control, $p > 0.5$; $n = 2$ slices), indicating that extracellular calcium influx is not required for wave propagation. To test whether intracellular calcium is required, we instead depleted intracellular calcium stores by incubating slices with either thapsigargin (1 μM) or cyclopiazonic acid (CPA, 30 μM). Under these conditions, stimulated calcium waves were dramatically reduced, demonstrating a critical role for the release of calcium from internal stores (Figures 6A and 6B; thapsigargin distance: 16.3% ± 10.2% of control, $p < 0.01$; thapsigargin wave amplitude: 36.8% ± 5.7% of control, $p < 0.05$, $n = 8$ slices; CPA distance: 1.8% ±1.8% of control, $p < 0.05$; CPA wave amplitude: 19.5% ± 2.0% of control, $p > 0.07$, $n = 2$ slices).

$P2Y_1$ receptor activation is known to initiate a G_q-phospholipase C (PLC)-linked metabotropic pathway resulting in increased IP_3 production and release of calcium from IP_3-gated stores (Berridge, 1995; Neary et al., 1996). Having demonstrated a crucial role for internal calcium release in the VZ waves, we next tested whether ryanodine- or IP_3- sensitive calcium stores were specifically involved by incubating slices with either ryanodine (20 μM), which inhibits calcium release from ryanodine-sensitive stores, or 2-APB (100 μM), which blocks IP_3-sensitive release. While ryanodine had no significant effect (ryanodine distance: 143.6% ± 33.4% of control, $p > 0.2$; ryanodine wave amplitude: 124.2% ± 11.7% of control, $p > 0.1$; $n = 4$ slices), 2-APB significantly decreased the waves (2-APB distance: 21.4% ± 4.5% of control, $p < 0.001$; APB wave amplitude: 28.2% ± 4.5% of control, $p < 0.0001$; $n = 9$ slices), supporting a role for IP_3-sensitive stores (Figure 6A).

The demonstrated need for IP_3-mediated calcium release in VZ waves implicates the PLC pathway as a part of their underlying mechanism. We therefore tested the involvement of PLC itself, which is also activated by

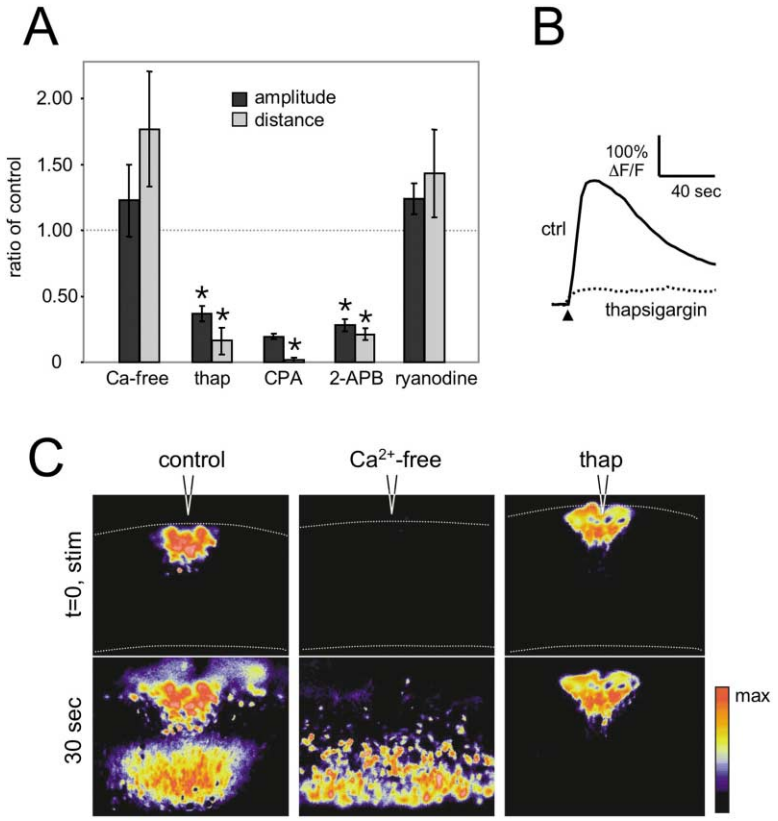

Figure 6. Waves Require IP_3-Mediated Calcium Release but Not Extracellular Calcium

(A) Depletion of intracellular calcium stores with thapsigargin (thap, 1 μm) or CPA (30 μm) or inhibition of IP_3-mediated intracellular calcium release with 2-APB (100 μm) abolishes stimulated VZ calcium waves. On the other hand, robust waves propagate under calcium-free conditions (with 2 mM EGTA) or inhibition of ryanodine-sensitive intracellular calcium release with ryanodine (20 μm). Histograms are represented as average ratios of experimental waves normalized to a paired control wave within the same slice.

(B) Raw trace of average ΔF/F shows a calcium wave that was abolished in the presence of thapsigargin (1 μM).

(C) Stimulation-associated calcium increases can be dissociated from VZ calcium waves. (Subtracted, pseudocolored images.) A calcium wave stimulated under control conditions is shown in the left column of panels, with stimulation (t = 0, upper) and 30 s (lower panel) time points. Electrode position is shown schematically above top image. Note the rapid calcium influx next to the electrode (t = 0, upper panel) and the slower development of a calcium wave in the underlying VZ in control conditions. The rapid calcium increase near the electrode, associated with stimulation, can be selectively prevented (center panels) by the removal of extracellular calcium (with 2 mM EGTA). VZ waves, however, are selectively blocked (right panels) by depletion of intracellular calcium stores (thapsigargin, 1 μm).

$P2Y_1R$, by using the PLC inhibitor U73122. Incubation with U73122 (5 μM) attenuated the waves (U73122 amplitude: 59.2% ± 19.3% of control, n = 4). However, incubation of pertussis toxin (PTX), a potent inhibitor of the separate adenylate cyclase pathway, had no effect (PTX amplitude: 101.6% ± 16.1% of control, n = 3). These data indicate that the downstream signaling pathway required for wave propagation involves the G_q-coupled metabotropic ATP receptor $P2Y_1$, activation of PLC, and release of calcium from IP_3-mediated intracellular stores.

Manipulating various calcium sources allowed us to dissect the mechanisms involved in the initiation of stimulated VZ waves. During electrically or mechanically stimulated waves, we observed a rapid calcium increase in cells at the stimulation site, within the first frame captured following stimulation (Figure 6C, left column, n = 147). We found that this rapid calcium increase was dependent upon extracellular calcium, because no increases were observed near the stimulating electrode when calcium was removed from the extracellular bath (Figure 6C, center column, n = 9), while typical fast increases were observed near the electrode when internal calcium stores were depleted (Figure 6C, right column, n = 10). This indicates that stimulation near the electrode caused a rapid calcium influx from the extracellular space, which may be due to depolarization and activation of calcium-permeable channels and/or electroporation of cells within the vicinity of the electrode. The subsequent, slower VZ waves, on the other hand, propagate robustly through the VZ in a calcium-free medium (Figure 6C, center, n = 9), indicating that they do not require extracellular calcium. VZ waves are instead abolished under conditions that deplete internal calcium stores (Figure 6C, right, n = 10). These experiments demonstrate a dissociation between the mechanism for immediate calcium increases near the electrode, which depend on external calcium, and the mechanism for the VZ waves themselves, which instead require release of calcium from internal stores. Spontaneous waves also propagate freely in a calcium-free environment. Importantly, these results indicate that extracellular calcium is not required for wave initiation or wave propagation.

Calcium Waves May Regulate Cell Cycle in the VZ

We demonstrate here that calcium waves propagate through cortical radial glial cells, which comprise the predominant population of proliferating progenitor cells in the embryonic VZ (Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004). Several aspects of our findings raise the intriguing possibility that the waves we observe in radial glial cells could be involved in cell proliferation. First, calcium transients during G_1 and M phase are known to affect the cell cycle in many types of dividing cells (Berridge, 1995; Berridge et al., 2000). Second, the ATP signaling pathway, which is required for VZ wave propagation, has been well documented to increase proliferation in a number of cell types, including glia (reviewed in Neary et al., 1996; Moll et al., 2002) and

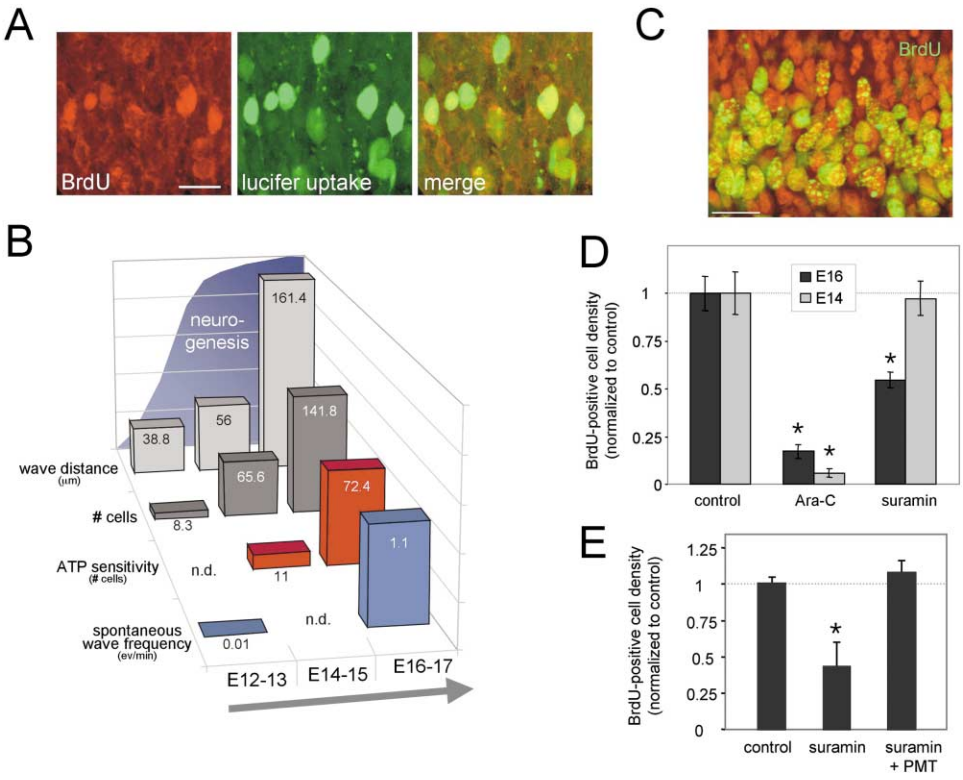

Figure 7. Radial Glial Calcium Waves May Be Involved in VZ Proliferation

(A) Cells that take up Lucifer yellow appear to be in S phase of the cell cycle. BrdU immunostain of VZ cells reveals several BrdU-positive, Lucifer yellow-positive cells. Scale bar, 20 μm.

(B) Developmental profile displaying changes in wave dynamics during the period of increasing neurogenesis in the VZ. In this three-dimensional graph, time is displayed along the x axis. Stimulated waves travel further (light gray bars) and involve more cells (dark gray bars) at later stages as shown. ATP sensitivity in the VZ also increases during this period (red bars). (E14 time point is with 100 μM, while E16 time point used 1 μM.) Spontaneous wave frequency also increases during the period of neurogenesis (blue bars). See text for numbers and error bars. (n.d., no data for this time point.)

(C) BrdU immunostain (green) with concanavalin A cellular stain (red) shows BrdU-positive VZ cells that are presumably in S phase of the cell cycle. Such images were used for quantification of proliferation experiments. Scale bar, 30 μm.

(D) In organotypic slice culture, the ATP receptor antagonist suramin (50 μM) significantly decreased VZ cell proliferation at E16 (dark gray bars), when VZ calcium waves are robust, shown as the percentage of BrdU-positive cells (normalized to control) that incorporated BrdU in S phase during a 1 hr pulse. However, at E14 (light gray bars), when waves are small and ATP sensitivity in the VZ is very low, suramin had no effect on proliferation. As expected, proliferation is decreased at both ages in the presence of cytosine arabinoside (Ara-C, 20.5 μM), an antimitotic agent.

(E) The phospholipase C activator PMT (200 ng/mL) rescues the antiproliferative effect of suramin.

progenitor cells (Sugioka et al., 1999; Uckermann et al., 2002; Scemes et al., 2003). In fact, proliferation can be stimulated by independent activation of several specific components that we show here to be required for VZ waves, including ATP, the $P2Y_1$ receptor, PLC, and IP_3-mediated calcium release (Neary et al., 1996; Berridge, 1995; Pearson et al., 2002; Sanches et al., 2002). Third, cell cycle in the VZ can be directly manipulated by inhibition of connexin channels (Bittman et al., 1997; Goto et al., 2002), which appear to be involved in spontaneous wave initiation. Specifically, connexin channel blockage significantly decreases entry into S phase and leads to a reduction in VZ proliferation (Bittman et al., 1997; Goto et al., 2002). Finally, the VZ calcium waves travel largely through radial glial cells—neuronal progenitor cells undergoing robust cell proliferation at E16 (Noctor et al., 2002). We therefore explored whether calcium waves could be involved in the regulation of VZ cell proliferation.

We first closely examined the cells involved in the initiation of VZ waves. As described above, VZ cells were observed to fill with small extracellular dyes, possibly via spontaneous openings of hemichannels, and we argue that these cells may be involved in the initiation of calcium waves. Interestingly, we noticed that the dye-loaded cells were often located in the upper third of the VZ, which, as a result of interkinetic nuclear migration, represents the S phase zone (Figure 2B). VZ cell progression through S phase can be assessed by monitoring DNA synthesis with a short pulse of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) (Caviness et al., 1995; LoTurco et al., 1995; Haydar et al., 1999; Haydar et al., 2000). Thus, we added BrdU to the extracellular medium during the 30 min Lucifer yellow incubation period to test whether cells involved in wave initiation were S phase cells. Coimmunolabeling with BrdU indicated that many of these cells were undergoing DNA synthesis (Figure 7A). This suggested that those cells responsible

for initiating spontaneous calcium waves may be part of a dividing cell population and that the calcium waves may be initiated in a cell cycle-dependent manner.

To explore further whether calcium waves could be involved in VZ cell proliferation, we tested if the expression of the waves matched the developmental period of neurogenesis. The rat cerebral cortex forms progressively from around E12 until birth, with levels of neuronal production peaking at around E16 (Bayer and Altman, 1991; Takahashi et al., 1996). The VZ cell cycle does not stay fixed during this time; in fact, its length is known to increase throughout the neurogenic period due to an increase in the length of G_1 (Takahashi et al., 1995). Paralleling this lengthening, cell divisions progress from symmetric proliferative divisions to asymmetric neurogenic divisions to terminal symmetric neurogenic divisions, resulting in an increased neuronal output from the VZ over time (Caviness et al., 1995) until neurogenesis is complete at around E19 (Bayer and Altman, 1991; Caviness et al., 1995). The mechanisms underlying this progression are not well understood. We reasoned that if the calcium waves were involved with regulation of VZ proliferation, wave dynamics may also change progressively during the period of neurogenesis.

We tested slices from earlier and later stages of cortical development and found that VZ calcium waves developed progressively during the neurogenic interval (Figure 7B). Stimulated waves were extremely small at E12, when G_1 is short and VZ cell divisions are largely proliferative, involving only 8.3 $\pm$ 1.2 cells (dark gray bars; n = 5 hemispheres) and traveling only 38.8 $\pm$ 3.0 μm (light gray bars; n = 8 hemispheres). Waves were slightly bigger at E14 (56 $\pm$ 8.3 VZ cells participating; n = 5 hemispheres; distance traveled: 65.6 $\pm$ 14.1 μm; n = 10 hemispheres) and increased significantly by E16, a stage at which most cell divisions produce neurons (141.8 $\pm$ 15.2 cells participating, $p < 0.005$; n = 5 hemispheres; distance: 161.4 $\pm$ 11.5 μm, $p < 0.0005$; n = 45 hemispheres). Waves could be stimulated until at least E20, although the thickness of the VZ decreases dramatically after E18, thus complicating quantitative wave analysis beyond that stage. We also found that the frequency of spontaneous waves increased dramatically from E13 (0.01 events/min/mm) to E16 (1.1 events/min/mm; blue bars, Figure 7B). Comparative dye uptake studies at E13 and E16 indicated that this change in wave frequency may be due to an increase in connexin hemichannel gating (Supplemental Figure S4 [http://www.neuron.org/cgi/content/full/43/5/647/DC1]). The developmental profile of the calcium waves thus parallels increasing levels of neuronal production in the cortical VZ (Takahashi et al., 1996) and places the VZ calcium waves in the right place at the right time to modulate VZ proliferation.

We next tested VZ cell sensitivity to ATP at different ages to determine whether it parallels the developmental profile of the calcium waves themselves. Indeed, at E14, when waves are significantly reduced in size, the VZ was much less sensitive to ATP (Figure 7B, red bars). Even a high concentration of ATP (100 μM) failed to produce large calcium responses at this early stage (11 $\pm$ 2.8 cells responding, n = 8 hemispheres). In sharp contrast, in the E16/E17 VZ, just 1 μM ATP produced a robust response (72.4 $\pm$ 11.2 cells responding, $p < 0.0001$, n = 5 hemispheres), and this increased ATP sensitivity persisted until at least E19. Thus, the sensitivity of VZ cells to ATP appears to develop coincidentally with the development of the calcium waves themselves. This developmental profile of wave activity and ATP sensitivity provides further evidence that the waves coincide with increasing levels of neuronal production in the VZ (Takahashi et al., 1996). In light of this evidence, we tested directly whether the calcium waves could be involved in the regulation of VZ proliferation.

We examined whether disruption of VZ calcium waves influences cell proliferation by culturing coronal slices from E16 rats in the presence of the ATP receptor antagonist suramin (50–100 μM), which abolishes coordinated wave activity but not individual calcium elevations in single cells (see Results above). Using BrdU immunohistochemistry in intact slices, we monitored DNA synthesis following a 1 hr BrdU pulse and fixation (Figure 7C). The addition of the ATP receptor antagonist significantly decreased BrdU labeling within the VZ, presumably reflecting decreased entry into S phase (Figure 7D, black bars; suramin: 54.7% $\pm$ 4.1% of control, n = 4 hemispheres; $p < 0.001$). Importantly, the antiproliferative effect of suramin could be rescued by simultaneous activation of the downstream IP_3 signaling pathway using pasteurella multocida toxin (PMT; 200 ng/mL), a PLC activator, arguing that the effect of suramin is specific to the calcium wave-activated PLC-IP_3 pathway (Figure 7E; suramin: 43.4% $\pm$ 16.9% of control, n = 8 hemispheres, $p < 0.005$; suramin + PMT: 108.0% $\pm$ 8.3% of control, n = 5 hemispheres). As expected, S phase progression in the presence of cytosine arabinoside (Ara-C, 20.5 μM), a cytosine analog that prevents DNA synthesis, was significantly disrupted as well (Figure 7D; 17.3% $\pm$ 3.6% of control, n = 6 hemispheres; $p < 0.0005$). The ability of ATP receptor antagonists to reduce VZ cell entry into S phase indicates that ATP signaling may affect proliferation within the developing cortex, as it does in other cell types (Neary et al., 1996; Pearson et al., 2002; Scemes et al., 2003). We next tested the effect of ATP receptor antagonists on proliferation at E14, when VZ waves are small and infrequent. We found no effect of suramin at this age (Figure 7D, gray bars; 97.5% $\pm$ 9.0% of control; n = 6 hemispheres), arguing that suramin's action at E16 is specific to the ATP-mediated calcium wave signaling pathway. Thus, one potential function of ATP-mediated calcium waves in the VZ may be to provide a temporal and spatial framework in which ATP can exert its mitogenic effect.

Discussion

We demonstrate here that spontaneous calcium waves propagate through the proliferative zone of the embryonic cortex. These waves are mediated by connexin hemichannels, extracellular ATP, the metabotropic ATP receptor subtype $P2Y_1$, and intracellular calcium release from IP_3-sensitive internal stores. Calcium waves spread selectively through the VZ, a region rich in radial glial cell bodies (Gotz et al., 2002; Noctor et al., 2002; Rakic, 2003), and we provide evidence that the majority of participating cells are radial glia. To our knowledge, this is the first demonstration of a calcium signaling mecha-

nism in cortical radial glial cells. We show that both VZ calcium waves and ATP sensitivity follow a developmental progression, becoming more robust from E12 to E16 and coinciding with increasing levels of neurogenesis (Takahashi et al., 1996). Further, cells involved in wave initiation appear to be in S phase of the cell cycle, and conditions that disrupt waves at E16 lead to a decrease in VZ cell entry into S phase, suggesting that waves may be involved in cortical proliferation. Since radial glial cells are known to act as neuronal progenitors (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001) and divisions in the VZ at this age are mostly asymmetric and neurogenic (Takahashi et al., 1996; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004), calcium waves may be involved in modulating neurogenesis during embryonic cortical development.

Connexin Hemichannels Initiate VZ Calcium Waves

Our data indicate a role for connexin hemichannels in the initiation of VZ calcium waves. Connexin hemichannels are formed by "undocked" individual gap junction channels located in the plasma membrane, allowing for communication between a cell's cytoplasm and the extracellular space (Cotrina et al., 1998; Hofer and Dermietzel, 1998; Cotrina et al., 2000; Stout et al., 2002; reviewed in Goodenough and Paul, 2003; Bennett et al., 2003). These hemichannels are believed to pass molecules smaller than ~1 kd; thus, brief channel openings, which occur under normal physiological conditions (Goodenough and Paul, 2003; Bennett et al., 2003), would readily allow passage of small charged molecules such as ATP (~0.5 kd). Consistent with a hemichannel-mediated initiation mechanism, VZ calcium waves were abolished in the presence of hemichannel inhibitors and increased dramatically in frequency upon lowering of extracellular calcium levels, a technique used to open connexin hemichannels. Furthermore, VZ cells became filled with a small dye (Lucifer yellow) when it was present in the extracellular space, and this dye fill was blocked by methods known to block hemichannels. Taken together, these results indicate that VZ cells express connexin hemichannels and suggest that radial glial calcium waves are hemichannel mediated. Interestingly, we found that dye-filled cells were often in S phase of the cell cycle, indicating that hemichannels are likely present in mitotically active progenitor cells in late G_1 or S phase.

Radial glial neuronal progenitor cells are robustly gap junction coupled (Lo Turco and Kriegstein, 1991; Bittman et al., 1997) and known to express high levels of connexins (Bittman and LoTurco, 1999; Nadarajah et al., 1997). The presence of connexin hemichannels adds complexity to the established role of gap junctions in development. In addition to being gap junction coupled to a VZ cluster, a radial glial cell may open connexin hemichannels, release ATP, and activate calcium increases in neighboring coupled or noncoupled cells. Interestingly, while radial glial cells express connexins throughout the cell cycle (Bittman et al., 1997; Nadarajah et al., 1997; Bittman and LoTurco, 1999), functional gap junction coupling decreases during G_1 and S phase (Bittman et al., 1997; Goto et al., 2002). Thus, in G_1 and S phase, most surface connexins may exist in the unapposed hemichannel form. As cells approach S phase and attempt to recouple, hemichannels may open in a regulated or spontaneous manner, thus initiating a local calcium wave. This is consistent with our dye uptake experiments showing that hemichannels appear to open in or near the S phase zone. Coupling and uncoupling of VZ precursor cells has been suggested to regulate entry into or exit from the cell cycle (Bittman et al., 1997; Bittman and LoTurco, 1999), supporting the possibility that spontaneous waves may function to modulate the cell cycle within clusters of neighboring VZ cells.

Calcium Waves in Proliferating Radial Glial Cells

Radial glial cells are key participants in the calcium waves that we describe here. To our knowledge, calcium wave signaling has not been previously identified in cortical radial glia, although oscillations have been observed in VZ cells (Owens and Kriegstein, 1998) and neurospheres (Scemes et al., 2003). In addition, cells in developing tissue such as the retinal VZ undergo both spontaneous calcium oscillations and waves (Wong et al., 1995; Catsicas et al., 1998; Pearson et al., 2002; Webb and Miller, 2003; Syed et al., 2004). Wave signaling allows for a coupling of spatial and temporal information, thus calcium waves have been proposed to play a role in mapping of neuronal networks (Katz and Shatz, 1996). However, the presence of calcium waves in developing epithelia such as the cortical VZ raises the intriguing possibility of regional specification and modulation at an earlier stage of development.

Our results suggest that ATP-mediated VZ calcium waves are involved in the modulation of radial glial cell proliferation. Extracellular ATP appears to have a mitogenic effect in the cortical VZ, consistent with studies of neurospheres (Scemes et al., 2003), astrocytes (Neary et al., 1996), and retina (Sugioka et al., 1999; Moll et al., 2002; Pearson et al., 2002; Uckermann et al., 2002). ATP acting at the $P2Y_1$ metabotropic ATP receptor activates the G_q-mediated IP_3 signaling pathway, which leads to increases in calcium that are required for cell proliferation (Berridge, 1995; Neary et al., 1996). In the VZ, ATP receptor antagonists appear to decrease proliferation by blocking coordinated wave activity without blocking calcium elevations in individual cells, thus ATP's mitogenic effect may spread as spatially and temporally controlled waves. Rather than globally increasing proliferation, however, the calcium waves likely play a more subtle role in the VZ.

One potential function for ATP-mediated calcium waves in the developing VZ could be to locally coordinate the cell cycle. Increases in intracellular calcium are required for entry into S phase and M phase (Berridge, 1995). In fact, at the G_1 transition, calcium may drive a cell to reenter S phase rather than exit the cell cycle as a postmitotic neuron (Berridge, 1995; Figure 8A). Since disruption of the VZ calcium waves leads to a decrease in S phase cells, one possible function of the waves may be to provide calcium increases that help initiate S phase in a nearby cluster of cells ("S phase coordination"). This possibility is further supported by our finding that VZ waves appear to be initiated by S phase cells.

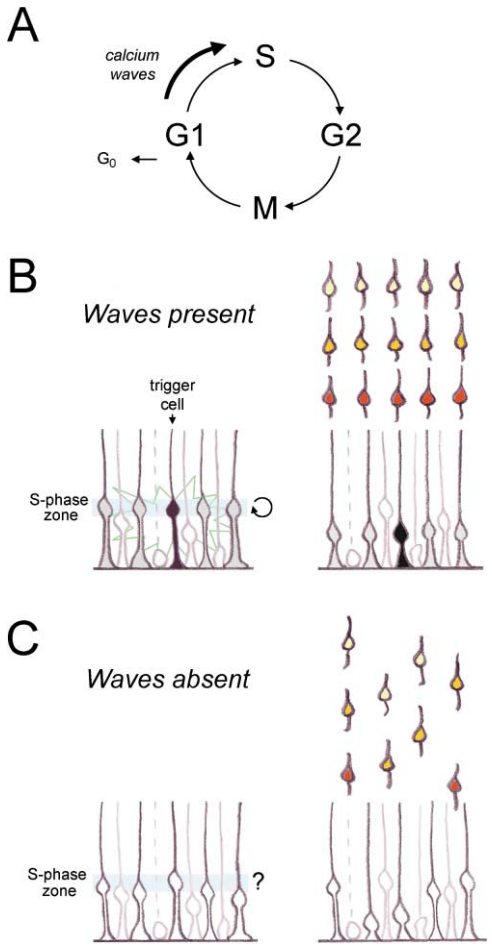

Figure 8. Potential Radial Glial Calcium Wave Model

Schematic illustrating a potential model for radial glial calcium waves in the VZ. (A) G_1 cells approaching S phase require an elevation in intracellular calcium before initiating DNA synthesis (Berridge, 1995). Calcium waves provide cells with an increase in intracellular calcium that may help drive cells toward S phase rather than exit from the cell cycle (G_0). (B) G_1 cells that are ready to enter S phase may linger in the S phase zone (light blue bar) before increasing intracellular calcium and beginning DNA synthesis. Activation by an ATP-mediated VZ wave (initiated by a nearby trigger cell in S phase, left panel) may drive a cohort of cells into S phase in a coordinated manner (gray cells; "S phase coordination"). This cohort may remain synchronized throughout the cell cycle (right panel), leading to the production of isochronic neurons (colored) that reside within the same cortical layer (but not necessarily within the same column). (C) In the absence of a synchronizing calcium wave, cycling VZ cells may lack coordinated initiation of calcium transients and thus may enter S phase more sluggishly. A cell may eventually increase calcium and begin DNA synthesis, but tight regulation of cell cycle within a cluster would be lost. Subpopulations of cells may prematurely differentiate and not reenter the cell cycle. Over time, asynchronous cell cycle may result in more random, decreased neuronal output from the VZ.

Radial glial cells in S phase may use ATP release to alert and recruit neighboring G_1 cells into S phase, thus driving local clusters through the cell cycle (Figure 8B). Speculating further, calcium waves could thus lead to synchronization of subpopulations of cycling cells (Cai et al., 1997) and coordinated neuronal production, which may increase the efficiency of neuronal output from the VZ (Figures 8B and 8C). Although individual spontaneous waves are limited to small clusters of cells, sequential waves were often generated in an array across the field (Figures 1G and 1H), raising the possibility that signals could be communicated throughout a hemisphere. The highly structured developing cortex may require precise communication both within and across proliferating regions. Future studies might test whether radial glial cell calcium waves could help to provide one pathway for this communication.

During VZ waves, we also observed calcium elevations in cells within the interphase and M phase zones. ATP-mediated calcium increases in these cells may result in different effects, such as gene activation (Gu and Spitzer, 1997) or M phase initiation, depending upon the precise timing of the signal. This hypothesis is particularly intriguing given that the calcium wave frequency increases throughout the neurogenic interval. Over time, changing calcium signals could lead to differential gene activation (Gu and Spitzer, 1997) or protein phosphorylation in VZ cells. Thus, in addition to providing a potential S phase coordination, calcium waves could provide "radial coordination" within the VZ. Tight regulation of cell cycle stages could allow the proliferating neuroepithelium to coordinate its remarkable timing and organization during development. Further detailed investigation could explore this and other possibilities.

Calcium waves also propagate through cultured astrocytes (reviewed in Haydon, 2001), and VZ waves share several pharmacological and kinetic features with these potentially related astrocytic waves. For example, astrocytic calcium waves are ATP mediated and involve P2YR signaling, connexin channels, and release of calcium from IP_3-sensitive stores (Haydon, 2001; Bennett et al., 2003; Goodenough and Paul, 2003). Furthermore, astrocyte waves propagate at a velocity similar to VZ waves (10.9 μm/s in the VZ versus 10–20 μm/s for astrocytes) (Giaume and Venance, 1998) and increase in frequency upon removal of extracellular calcium (Zanotti and Charles, 1997; Cotrina et al., 1998; Stout et al., 2002). Recent evidence demonstrates that astrocytes also release ATP through connexin hemichannels (Cotrina et al., 1998; Arcuino et al., 2002; Stout et al., 2002), although others report a role for wave spread through functional gap junctions per se (reviewed in Giaume and Venance, 1998; Haydon 2001). These mechanistic similarities are unlikely due to coincidence; one might expect these calcium waves to share similar mechanisms and possibly even related functions, since radial glia and astrocytes are lineage-related cell types (Alvarez-Buylla et al., 2001; Gotz et al., 2002).

Potential Radial Glial Interactions with Neurons

Radial glia generate and then guide newborn neurons into the cortical plate (Noctor et al., 2001). Therefore, radial glial cells are in intimate contact with neurons not only within the VZ, where neurons are born, but also throughout each layer of the developing cortex. A number of potential signaling pathways between radial glia and immature postmitotic neurons have been identified, including signaling through Notch (Gaiano et al., 2000), neuregulin (reviewed in Campbell, 2003), integrins (reviewed in Schmid and Anton, 2003), and Reelin (Camp-

bell, 2003; Hartfuss et al., 2003). VZ calcium waves may provide an additional pathway for direct communication between radial glia and neuronal progeny. Indeed, we observe some cells with neuronal morphology participating in the calcium waves. Our demonstration that a cocktail of glutamate, GABA, and glycine receptor antagonists attenuates VZ waves suggests that transmitters other than ATP may play a role in wave mediation, although they are not required. Low levels of these neurotransmitters are present endogenously in the developing cortex (LoTurco et al., 1995; Flint et al., 1998) and may be released from either immature neurons or radial glia themselves. For example, upon stimulation with ATP, radial glial cells may release glutamate that activates calcium transients in nearby migrating neurons, a mechanism that has been demonstrated for astrocytes signaling to mature neurons (see Haydon, 2001). Calcium increases in immature neurons on a time scale similar to those observed here have been suggested to coincide with bursts of locomotion necessary for neuronal migration (Behar et al., 1996; Komuro and Rakic, 1996). Therefore, in addition to their other demonstrated functions, radial glial cells may provide crucial cues for migrating neuronal progeny. Spatially restricted VZ calcium waves that propagate among dividing neuronal progenitor cells could for example lead to an early specification of neonatal neuronal domains (Yuste et al., 1992), whose size (50–120 μm) roughly matches the extent of a spontaneous VZ wave (~75 μm), although there is evidence that some migrating neurons travel tangentially away from their birthplace (e.g., Walsh and Cepko, 1992).

The function of radial glial cells in cortical development is more dynamic than was once envisioned, extending well beyond their traditional role in supporting neuronal migration. Radial glia are key neuronal progenitors (Malatesta et al., 2000; Alvarez-Buylla et al., 2001; Noctor et al., 2001, 2002; Malatesta et al., 2003; Anthony et al., 2004), producing clones of neuronal progeny that then migrate along radial glial fibers into the cortex (Noctor et al., 2001; Rakic, 2003). Our new findings provide evidence for a dynamic radial glial calcium signaling process, indicating that radial glial cells are capable of signaling to each other via calcium waves that may regulate neuronal production. Radial glia appear to be equipped with a complex set of "calcium signaling tools" (Berridge et al., 2000) and can respond within seconds to cues around them. Developmentally regulated calcium waves may provide a mechanism for modulating radial glial cell cycle or specifying local regions of the VZ. Calcium waves are also activated by mechanical stimulation, suggesting that perturbations in the embryonic environment could activate propagating waves in utero and possibly lead to subtle alterations in neurogenesis. The demonstration of calcium waves in radial glial cells indicates that this cell type shares signaling mechanisms with astrocytes. Considering that both radial glia and astrocytes can function as neuronal stem cells (Malatesta et al., 2000; Alvarez-Buylla et al., 2001; Noctor et al., 2001), radial glial calcium waves could have important implications for neurogenesis and cortical architecture in the developing and adult brain.

Experimental Procedures

Pharmacological Agents

Extracellular artificial cerebrospinal fluid (ACSF) solution containing NaCl (125 mM), KCl (5 mM), NaH_2PO_4 (1.25 mM), $MgSO_4$ (1 mM), $CaCl_2$ (2 mM), $NaHCO_3$ (25 mM), and glucose (20 mM) at pH 7.4, 25°C, and 310 mOsm/L was bubbled with 95% O_2/5% CO_2. For most spontaneous imaging experiments, $CaCl_2$ was replaced with ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, 2 mM, SIGMA, St. Louis, MO, pH adjusted to 7.4). For experiments done in low pH, ACSF was bubbled with 100% CO_2 to maintain final pH ~6.5. All experiments were done at room temperature (~25°C). All pharmacological agents used are listed in the Supplemental Data (http://www.neuron.org/cgi/content/full/43/5/647/DC1).

Tissue Preparation

All animals used were cared for according to protocols approved by the Institutional Animal Care and Use Committee at Columbia University. Timed pregnant Sprague Dawley rats (Taconic, NY) at embryonic days E14 to E18 were deeply anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg, Henry Schein, Port Washington), uterine horns were exposed, and embryos removed. Following rapid decapitation, brain was removed in cold ACSF and placed into low-melt agarose (Fisher Biotech, Fair Lawn, NJ). Agarose was allowed to solidify on ice (2–3 min), and block was sectioned coronally at 350–400 μm in cold ACSF using a Leica VT100S vibrating blade microtome (Nussloch, Germany). Slices were placed in ACSF bubbled with 95% CO_2 and 5% O_2 and recovered at room temperature. For cortical explants and partial brain preparations, ventral structures were dissected away from whole embryonic brains in cold ACSF, and tissue was recovered at room temperature.

Calcium Imaging

After recovering for 30 to 60 min, slices or explants were transferred to oxygenated ACSF containing the acetoxymethylester form of the calcium indicator Fluo-3 (Fluo-3AM, 10–14 μM, Molecular Probes, Eugene, OR) and loaded in the dark 1–3 hr. Details of image acquisition are listed in the Supplemental Data (http://www.neuron.org/cgi/content/full/43/5/647/DC1). Preparations were stimulated using an extracellular tungsten microelectrode (1 MΩ, 1 μM tip diameter, World Precision Instruments, Inc., Sarasota), usually in the MZ, with a 100 μs current pulse of 200–800 μA. Each wave and paired control were stimulated at the same current amplitude. For mechanical stimulation, the tip of the electrode was displaced by 1–5 μm. Drugs were applied either to the bath or directly via a 100 or 200 μM wide drug applicator (ALA Scientific Instruments, Westbury). For dye uptake experiments, slices or cortical explants were equilibrated for 1–3 hr in ACSF, then incubated in (1 mM) Lucifer yellow or Alexa-594 hydrazide for 30 min, washed, and fixed. In whole-cell recording experiments, cell morphology was considered "radial glial" if cell body was bipolar with endfoot contacting ventricle and radial process reaching the pia, "neuronal" if cell body appeared bipolar with short leading and trailing processes, and "other" if cell did not fall into either the radial glial or neuronal category. Due to mechanical stimulation of cells during cell-attached recording, responses to ATP were sometimes desensitized, thus cells that did not respond to ATP application could not be quantified accurately. Data are shown for cells with positive responses only.

Electrophysiology and Data Analysis

See the Supplemental Data (http://www.neuron.org/cgi/content/full/43/5/647/DC1) for all details regarding electrophysiological recordings and data analysis.

Immunohistochemistry

E16 embryos were perfused transcardially with cold phosphate-buffered saline (PBS, GIBCO, Rockville, MD) followed by cold 4% paraformaldehyde (PFA) in PBS, brains removed, and postfixed overnight. Brains were cryostat sectioned (10–30 μm) or sliced in agarose (100–200 μm) using a Vibratome (Ted Pella, St. Louis). Slices were incubated in blocking solution for 1 hr (10% normal goat serum, 0.1% Triton X-100, and 0.2% gelatin in PBS), then incubated 24–48 hr in primary antibody diluted in buffer (2% normal goat serum,

0.1% Triton X-100, and 0.04% gelatin in PBS). Primary antibodies included fluorescein-conjugated rat monoclonal anti-BrdU (1:10; Accurate Chemical, Westbury) and rabbit polyclonal anti-$P2Y_1$ (0.6 mg/ml stock diluted 1:200; Chemicon, Temecula). Slices were then washed three times for 10 min in PBS, incubated for 0.5–1 hr in secondary antibody diluted in buffer, and washed in PBS (five times for 10 min). Cy3-conjugated goat anti-rabbit (0.75 mg/ml stock diluted 1:50; Jackson ImmunoResearch Laboratories, West Grove) was used as a secondary antibody, and concanavalin A (0.02 mg/mL; Molecular Probes) was used to help determine cell density.

Slice Proliferation Assay

Live slices (350 μm) were prepared from E16 rats as described above and placed onto Millicell-CM culture inserts (Millipore, Bedford) in 6-well culture trays (Corning, Inc., Corning) with medium containing 25% Hanks balanced salt solution, 47% Basal modified Eagle's medium, 25% normal horse serum, 1% Pen/Strep/Glutamine (GIBCO), and 0.66% glucose (Collaborative Biomedical, Bedford). Slices were incubated for 16 hr at 37°C with 95% O_2, 5% CO_2. One hour before fixation, 2.5 mg/ml 5-Bromo-2′-deoxyuridine (BrdU, SIGMA, in 0.9% NaCl) suramin (50–100 μM), pasteurella multocida toxin (PMT, 200 ng/mL), and/or cytosine arabinoside (Ara-C, 20.5 μM; Calbiochem) were added to the culture medium. Slices remained in the incubator for 60 min, were fixed with PFA, processed for BrdU incorporation, and imaged. Please see the Supplemental Data (http://www.neuron.org/cgi/content/full/43/5/647/DC1) for details of imaging and quantification.

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